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Rapid Detection of Chloramphenicol Resistance in *Haemophilus influenzae*

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We compared a rapid (1-h) screening method for the detection of chloramphenicol acetyltransferase (CAT) activity with the standard spectrophotometric CAT assay to determine whether CAT-mediated chloramphenicol resistance in *Haemophilus influenzae* could be determined upon primary isolation. Of 58 *H. influenzae* cell sonicates, 28 had detectable CAT activity when the chloramphenicol-dependent production of free coenzyme A from acetyl coenzyme A was measured spectrophotometrically (standard method). These 28 strains were identified as producing CAT by the rapid method which uses lysed cell suspensions and a color change to detect CAT. The remaining 30 strains did not have CAT activity detectable by either method. This 1-h test for CAT should prove to be useful for the early presumptive identification of chloramphenicol resistance in *H. influenzae*.

Haemophilus influenzae causes serious infections in children, notably meningitis and epiglottitis. The emergence of ampicillin resistance in *H. influenzae* in 1974 (4) and its spread through the population have necessitated the use of chloramphenicol in the initial antibiotic therapy of children with systemic infections (2). In 1976, the first chloramphenicol-resistant strain was isolated (11). Recently, multiresistant strains carrying resistance to chloramphenicol, tetracycline, and ampicillin have been described (1, 6, 8). Unfortunately, the incidence of chloramphenicol-resistant *H. influenzae* has increased. In 1980, 1% of the strains received by the Centers for Disease Control (J. Band, personal communication) and 3% in a recent Swedish survey (12) were found to be resistant to chloramphenicol. The majority of strains owe their resistance to the production of chloramphenicol acetyltransferase (CAT) (8).

A reliable method requiring a minimal amount of time and equipment is needed for the detection of chloramphenicol-resistant *H. influenzae*. In this report we describe a 1-h screening test which detects the production of CAT in *H. influenzae*.

MATERIALS AND METHODS

Bacterial strains. Fifty-eight *H. influenzae* strains were examined. Of these, 30 were collected or referred to us because of putative chloramphenicol resistance. The remaining 28 strains were isolated from the blood or cerebrospinal fluid of infants hospitalized at Children's Orthopedic Hospital and Medical Center between January and December 1980. Fourteen of the strains did not agglutinate in Difco polyvalent (a through f) typing sera and were considered nontypable (unencapsulated). The remaining 16 strains reacted with Difco polyvalent and type b sera, but not with any of the other five typing sera (a, c, d, e, and f), and were considered type b.

Media. The solid medium used for growth of *H. influenzae* was 3.5% brain heart infusion (Difco Laboratories) supplemented with 10 μg of hemin, 10 μg of L-histidine, and 2 μg of nicotinamide adenine dinucleotide per ml. For minimal inhibitory concentration (MIC) determinations, the solid medium was supplemented with 1, 2, 5, 10, and 20 μg of chloramphenicol per ml. Brain heart infusion broth (3.5%) supplemented with 10 μg of hemin, 10 μg of L-histidine, and 2 μg of nicotinamide adenine dinucleotide per ml was used to grow the organisms for inoculation determinations. Plate cultures, except for those used for the MIC determinations, were grown at 36.5°C in 5% CO_2, whereas liquid cultures were incubated at 37°C and shaken at 200 rpm. The plates used for the MIC determinations were incubated at 37°C without added CO_2.

Determinations of MICs. MICs were determined by agar dilutions, using a Steers replicator. An inoculum of 10^5 was used as described by Syriopoulou et al. (10).

Spectrophotometric CAT assay. Extracts were prepared from all *H. influenzae* strains as described by Zaidenzaig and Shaw (14). As previously described (8), CAT activity was assayed spectrophotometrically by determining the chloramphenicol-dependent appearance of coenzyme A, using acetyl coenzyme A as the substrate.

Rapid CAT assay. The strains were grown overnight on solid media. A heavy loopful of organisms was suspended in 0.2 ml of 1 M NaCl–0.01 M ethylenediaminetetraacetic acid–0.05% sodium dodecyl sulfate (pH 8.0). The suspension was incubated at 37°C for 60 min. At the conclusion of incubation, the solution was slightly turbid. Duplicate tubes were prepared with each strain. To each tube was added 0.2 ml of a solution containing 2 parts 0.2 M tris(hydroxymethyl)-aminomethane (adjusted to pH 8.0 at 37°C), 2 parts 5 mM acetylcoenzyme A, and 1 part 10 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) in 0.1 M tris-(hydroxymethyl)aminomethane (pH 8.0). A 0.2-ml amount of 5 mM sterile chloramphenicol (dissolved in water) was added to one tube (test reaction), and an equivalent amount of water (0.2 ml) was added to the duplicate tube (control). The tubes were reincubated at 37°C for 5 min, and the reaction was evaluated by comparing the color in the control tube with that in the experimental tube. A color range from pale yellow to deep yellow indicates CAT activity. This color change is due to the reaction of DTNB with the free sulfhydryl group of coenzyme A (Ellman reaction) (3). CAT found in enteric bacteria produced diacetylated chloramphenicol by a two-step process; 2 mol of free coenzyme A is generated for each mole of chloramphenicol that is inactivated (9). It has not been determined whether the *H. influenzae* enzyme catalyzes the second reaction. The two reactions are as follows: (i) CAT + chloramphenicol + acetyl-*S*-coenzyme A → chloramphenicol-3-acetate + hydrogen sulfide-coenzyme A and (ii) chloramphenicol-3-acetate + acetyl-*S*-coenzyme A → chloramphenicol-1,3-diacetate + hydrogen sulfide-coenzyme A.

We were unable to detect CAT activity with lower concentrations of sodium dodecyl sulfate with 24 h of incubation at 37°C. Higher concentrations of sodium dodecyl sulfate, producing clear lysates, did not have detectable CAT activity.

RESULTS

MICs were performed on all strains as described above. A total of 30 (14 unencapsulated and 16 type b) isolates had MICs of ≥10 μg/ml and were considered resistant to chloramphenicol. The remaining strains had MICs of ≤2 μg/ml and were considered susceptible to chloramphenicol. No chloramphenicol-susceptible strains had measurable CAT activity; 28 of 30 chloramphenicol-resistant strains had CAT activity. Similar results were found when the rapid assay was performed. The same two isolates, one an unencapsulated strain and the other a type b strain, appeared to have no CAT activity in either assay, but were chloramphenicol resistant.

No difference in results was found when capsulated type b strains were compared with unencapsulated strains.

DISCUSSION

The majority (more than 99%) of the chloramphenicol-resistant gram-negative organisms produce CAT, which chemically inactivates chloramphenicol (13). Similarly, we have found that 93% of the chloramphenicol-resistant *H. influenzae* strains (MIC ≥ 10 μg/ml) produce a detectable CAT with the spectrophotometric assay. This assay requires 2 days of preparation, a spectrophotometer, and highly trained personnel. As a result, this procedure is not practical to run in many routine clinical laboratories. In this report we describe a 1-h assay which requires a minimal amount of equipment and can be easily performed by either technicians or medical technologists working in either a research or a clinical setting. The rapid method gave identical qualitative results when compared with the spectrophotometric assay.

Both CAT assays use DTNB in the reaction mixture. Certain enteric chloramphenicol-inactivating enzymes are inhibited by DTNB. Type II enzymes are more sensitive to DTNB than is type I or III (13, 14). Therefore, to eliminate the possibility that the two isolates which had high MICs (40 to 50 μg/ml) but were negative in both CAT assays did not produce a DTNB-sensitive enzyme, these strains were retested with a radioactive assay. This procedure does not use DTNB and is 16-fold more sensitive than the spectrophotometric assay in the laboratory (9). Neither strain had detectable CAT activity (data not shown), suggesting that these two strains owe their resistance to some other mechanism.

Two enteric R plasmids have been described which confer resistance to chloramphenicol by decreasing cellular permeability to chloramphenicol; these strains lack detectable CAT (7). A present our two strains lacking detectable CAT activity are being examined to determine the mechanism of resistance.

The rapid assay detected over 90% of all chloramphenicol-resistant *H. influenzae* strains tested and 100% of all strains which produced CAT; all chloramphenicol-susceptible strains lacked the enzyme. We conclude that this assay will detect the majority of chloramphenicol-resistant *H. influenzae* strains and could easily be performed in conjunction with one of the rapid methods used to detect beta-lactamase activity.

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LITERATURE CITED

1. **Bryan, L. E.** 1978. Transferable chloramphenicol and ampicillin resistance in a strain of *Haemophilus influenzae*. Antimicrob. Agents Chemother. **14**:154–156.
2. **Center for Disease Control.** 1974. Ampicillin resistant *Haemophilus influenzae* meningitis: Maryland Georgia. Morbid. Mortal. Weekly Rep. **23**:77–78.
3. **Dietz, A. A., and H. M. Rubinstein.** 1972. Standardization of the Ellman reaction. Clin. Biochem. **5**:136–138.

4. **Elwell, L. P., J. De Graaff, D. Seibert, and S. Falkow.** 1975. Plasmid-linked ampicillin resistance in *Haemophilus influenzae* type b. Infect. Immun. **12**:404–410.
5. **Kattan, S.** 1976. Chloramphenicol resistant *Haemophilus*. Lancet **i**:814.
6. **Kenny, J. F., C. D. Asburg, and R. H. Michaels.** 1980. Meningitis due to *Haemophilus influenzae* type b resistant to both ampicillin and chloramphenicol. Pediatrics **66**:14–16.
7. **Nagai, Y., and S. Mitsuhashi.** 1976. New type of R factors incapable of inactiviting chloramphenicol. J. Bacteriol. **109**:1–7.
8. **Roberts, M. C., C. D. Swenson, L. M. Owens, and A. L. Smith.** 1980. Characterization of chloramphenicol-resistant *Haemophilus influenzae*. Antimicrob. Agents Chemother. **18**:610–615.
9. **Shaw, W. V.** 1975. Chloramphenicol acetyltransferase from chloramphenicol-resistant bacteria. Methods Enzymol. **43**:737–755.
10. **Syriopoulou, V. Ph., D. W. Scheifele, C. M. Sack, and A. L. Smith.** 1979. Effect of inoculum size on the susceptibility of *Haemophilus influenzae* type b to beta-lactam antibiotics. Antimicrob. Agents Chemother. **16**:510–513.
11. **Van Klingeren, B., J. D. A. van Embden, and M. Dressens-Kroon.** 1977. Plasmid-mediated chloramphenicol resistance in *Haemophilus influenzae*. Antimicrob. Agents Chemother. **11**:383–387.
12. **Zackrissen, G., and J. E. Brorson.** 1980. Antibiotic sensitivity of *Haemophilus influenzae* strains including three recent chloramphenicol resistant isolates. Acta Pathol. Microbiol. Scand. Sect. B **88**:193–198.
13. **Zaidenzaig, Y., J. E. Fitton, L. G. Packmand, and W. V. Shaw.** 1979. Characterization and comparison of chloramphenicol acetyltransferase variants. Eur. J. Biochem. **100**:609–618.
14. **Zaidenzaig, Y., and W. V. Shaw.** 1976. Affinity and hydrophobic chromatography of three variants of chloramphenicol acetyltransferase specified by R factors in *Escherichia coli*. FEBS Lett. **62**:266–271.